AD_____

Award Number: W81XWH-06-1-0533

TITLE: Inflammatory Markers and Breast Cancer Risk

PRINCIPAL INVESTIGATOR: Brenda Diergaarde, Ph.D.

CONTRACTING ORGANIZATION: University of Pittsburgh
Pittsburgh, PA 15260

REPORT DATE: July 2008

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-07-2008	Annual	1 Jul 2007 - 30 Jun 2008

4. TITLE AND SUBTITLE

Inflammatory Markers and Breast Cancer Risk

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0533

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Brenda Diergaarde, Ph.D.

E-Mail: diergaardeb@upmc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Pittsburgh
Pittsburgh, PA 15260

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Mammographic breast density is one of the strongest risk factors identified for breast cancer, and a marker of cancer risk for both breasts. To gain further insight into the role of inflammatory cytokines in the etiology of breast density, this study investigates associations between serum cytokine levels, genetic variation in cytokine genes, and breast density. This report provides information on the progress made during the second year of the grant. It should be noted that only in January 2008 Dr. Diergaarde officially became the PI of this award and that no monies could be spent until this change in PI was official. This did affect our progress this year. A study specific database was created and we have started analyzing the serum cytokine data received from Dr. Tracy's laboratory. SNPs were selected for genotyping: candidate functional SNPs were identified from the literature and databases such as SeattleSNPs; tagSNPs were selected using data from HapMap and the HaploView/Tagger program. We are currently genotyping the MAMS samples using the iPLEX Gold assay (Sequenom).

15. SUBJECT TERMS
Breast cancer; breast density; cytokines; genetic variation

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	9	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

INTRODUCTION

Mammographic breast density is one of the strongest risk factors identified for breast cancer, and a marker of cancer risk for both breasts (1, 2). Information on the etiology of breast density is currently limited. Various evidence suggest that exposure to sex hormones, estrogens in particular, may be an important factor. Changes in density have been observed in response to hormone replacement therapy use and use of tamoxifen (3, 4). Pro-inflammatory cytokines, specifically tumor necrosis factor (TNF)-α and interleukin (IL)-6, have emerged as critical regulators of estrogen synthesis in breast tissues (5), and may also affect breast density and breast cancer risk. To gain further insight into the role of inflammatory cytokines in the etiology of breast density, this study investigates associations between serum cytokine levels, genetic variation in cytokine genes, and breast density. Existing data and banked specimens from women who participated in a recently completed, cross-sectional study on hormones and breast density, the Mammograms and Masses Study (MAMS), are used.

BODY

- **IL-6, soluble IL-6R and TNF-α serum levels and breast density**

Serum levels of IL-6, soluble IL-6R and TNF-α were measured for all 722 study participants. IL-6 and soluble IL-6R were both measured using ELISA, TNFα was measured using Luminex. The lower limit of detection for the IL-6 assay is 0.156 pg/mL, and we observed an average intra-assay coefficient of variation (CV) of 16.0%; the lower limit of detection for the IL-6 sR assay is 6.5 pg/ml and average intra-assay CV was 8.1%; the TNF-α assay could measure concentrations of TNF-α ≤3.2 pg/mL. Our reproducibility study demonstrated an average intra-assay CV of 10.8%.

The number of premenopausal women was relatively small, too small to meaningfully analyze separately. We excluded them from these preliminary analyses leaving a total of 543 participants: 145 women with benign breast disease and 348 well controls. Characteristics of the study population are presented in Table 1. Summary statistics for IL-6 and TNF-α serum levels, and breast density variables are shown in Table 2. Please note that we have not analyzed the IL-6 sR data yet.

Pearson's correlation was used to examine the correlation between IL-6 and TNF-α serum levels and percent breast density and dense breast area (Table 3). Linear logistic regression was used to further assess the relationship between the inflammatory markers and percent breast density and dense breast area. Unadjusted (not in table), age-adjusted, age- and BMI-adjusted, and fully adjusted models were fit for each combination of inflammatory marker and density variable. The multivariable model included adjustment for variables demonstrated to be associated with breast density and/or breast cancer in previous studies, see Table 4.

Table 1. Characteristics of the study population by status

	Benign Ntotal=145 N (%)	Well Ntotal=398 N (%)	P*
Age, years; mean (SD)	58.3 (7.4)	62.0 (8.1)	<0.001
<50	12 (8.3)	4 (1.0)	<0.001
50-59	68 (27.3)	181 (45.5)	
60-69	55 (37.9)	135 (33.9)	
≥70	10 (6.9)	78 (19.6)	
Ethnicity			0.94
White	136 (93.8)	374 (94.0)	
Other	9 (6.2)	24 (6.0)	
Body mass index, kg/m^2; mean (SD)	27.9 (0.5)	28.3 (0.3)	0.58
Normal, <25 kg/m^2	44 (30.6)	132 (33.2)	0.45
Overweight, 25-<30 kg/m^2	58 (40.3)	137 (34.4)	
Obese, ≥30 kg/m^2	42 (29.2)	129 (32.4)	
Age at menopause, years			<0.001
<50	85 (59.9)	165 (42.3)	
≥50	57 (40.1)	225 (57.7)	
Surgical menopause status			0.12
No hysterectomy	90 (65.7)	276 (72.4)	
Hysterectomy without oophorectomy	16 (11.7)	48 (12.6)	
Hysterectomy with uni- or bilateral oophorectomy	31 (22.6)	57 (15.0)	
Previous breast biopsy	60 (41.7)	57 (14.3)	<0.001
First degree relative with history of breast cancer	18 (12.5)	56 (14.2)	0.62
Ever been pregnant	121 (83.5)	333 (83.7)	0.95
Age at first pregnancy lasting ≥6 months			0.35
Never pregnant/no pregnancies ≥6 months	32 (22.1)	80 (20.1)	
<20	18 (12.4)	35 (8.8)	
20-24	52 (35.9)	143 (35.9)	
25-29	27 (18.6)	90 (22.6)	
≥30	15 (10.3)	50 (12.6)	
History of breastfeeding			0.85
Not applicable†	32 (22.2)	81 (20.4)	
No	57 (39.6)	156 (39.2)	
Yes	55 (38.2)	161 (40.5)	
Hormone therapy use status			<0.001
Never	27 (18.8)	140 (35.2)	
Former	43 (29.9)	204 (51.3)	
Current (within previous 3 months)	74 (51.4)	54 (13.6)	
Current NSAID use	30 (34.1)	194 (49.7)	0.01

*P values from t tests for continuous variables and chi square tests for categorical variables

†One participant reported a stillbirth after 6 months gestation, and was categorized as "not applicable" for history of breastfeeding

Abbreviations used: SD, standard deviation; NSAID, non-steroidal anti-inflammatory drug use

Table 2. Summary of IL-6 and TNF- α levels and breast density variables by status

	Benign				Well				P†
	N	Mean (SD)	Age-adjusted Transformed Mean*	Median	N	Mean (SD)	Age-adjusted Transformed Mean*	Median	
IL-6, pg/mL	145	2.67 (2.72)	2.12	1.97	398	2.89 (2.91)	2.17	1.97	0.76
TNF-α, pg/mL	145	3.00 (1.60)	2.68	2.59	395	2.98 (1.83)	2.62	2.67	0.68
Dense breast area, cm^2	145	48.0 (30.6)	42.8	44.6	397	40.9 (26.6)	36.1	36.7	0.02
Percent breast density, %	145	35.2 (18.8)	31.2	34.2	397	29.6 (19.4)	25.8	26.0	0.01

*Transformed mean is a geometric mean for the inflammatory markers and a mean calculated on the square root scale and back-transformed to the natural scale for breast density variables

†P values from ANOVA comparing distributions among benign breast disease to well controls using natural log transformations of the inflammatory markers and square root transformations of the breast density variables with adjustment for age

Table 3. Correlation between inflammatory markers and breast density variables by status*

	Benign			Well			P†
	N	ρ	P value	N	ρ	P value	
Dense breast area							
IL-6	145	-0.03	0.72	397	-0.06	0.24	0.77
TNF-α	145	0.04	0.64	394	-0.01	0.78	0.59
Percent breast density							
IL-6	145	-0.21	0.01	397	-0.20	<0.001	0.90
TNF-α	145	-0.11	0.19	394	-0.18	<0.001	0.44

*Calculated using Pearson's correlation coefficient with natural log transformation of the inflammatory markers and square root transformations of the breast density variables

†P values for comparison of correlation coefficients between benign breast disease and well control groups

Table 4. Results of regressions of breast density variables on inflammatory markers, by status

	Benign								
	Age-adjusted			Age- and BMI-adjusted			Multivariable Adjusted		
	N	β (SE)	P	N	β (SE)	P	N	β (SE)	P
Dense breast area									
IL-6	145	-0.13 (0.28)	0.64	144	-0.07 (0.31)	0.83	80	-0.44 (0.51)	0.39
TNF-α	145	0.18 (0.37)	0.63	144	0.26 (0.39)	0.50	80	0.46 (0.57)	0.42
Percent breast density									
IL-6	145	-0.55 (0.23)	0.02	144	-0.08 (0.23)	0.74	80	-0.41 (0.37)	0.28
TNF-α	145	-0.42 (0.31)	0.18	144	-0.03 (0.29)	0.92	80	0.05 (0.42)	0.90
	Well								
	Age-adjusted			Age- and BMI-adjusted			Multivariable Adjusted		
	N	β (SE)	P	N	β (SE)	P	N	β (SE)	P
Dense breast area									
IL-6	397	-0.16 (0.16)	0.32	397	-0.07 (0.17)	0.69	365	-0.13 (0.19)	0.47
TNF-α	394	-0.04 (0.23)	0.87	394	0.09 (0.24)	0.71	362	0.03 (0.26)	0.92
Percent breast density									
IL-6	397	-0.54 (0.14)	<0.001	397	-0.11 (0.14)	0.43	365	-0.12 (0.15)	0.41
TNF-α	394	-0.71 (0.20)	<0.001	394	-0.16 (0.19)	0.39	362	-0.17 (0.21)	0.41

*Regressions performed using natural log transformations of the inflammatory markers and square root transformations of the breast density variable

†Adjusted for age, race, body mass index category, age at menopause, surgical menopause status, history of breast biopsy, first degree relative with history of breast cancer, ever been pregnant, age at first pregnancy lasting ≥6 months, history of breastfeeding, hormone therapy use status, NSAID use, time between blood draw and mammogram

- **Measurements of soluble TNFRI and TNFRII**

We planned to use ELISA assays by R&D Systems to measure soluble TNFRI and TNFRII. However, we are having issues with these assays (unreproducible results) and therefore the assays are currently on hold. We are investigating other assays.

- **Genetic variation**

To date, all buffy coat samples have been transferred to Dr. Robert Ferrell's laboratory, DNA has been isolated from these samples, and plates were prepared for genotyping. In total, 680 individuals will be genotyped, all Caucasian.

We selected potential functional SNPs and tagSNPs for the following genes: *IL6*, *IL6R*, *IL6ST* (gp130), *TNF-α*, *TNFRSF1A* (TNFRI), and *TNFRSF1B* (TNFRII). Candidate functional SNPs were identified from the literature and databases such as SeattleSNPs; tagSNPs were selected

using data from HapMap and the HaploView/Tagger program (MAF>0.05, r^2 >0.08). IL-6 acts by binding to IL-6R which must associate with gp130 in order for signal transduction to occur. Therefore, we also included the *IL6ST* gene.

We are currently genotyping the samples using the iPLEX Gold assay (Sequenom). This system utilizes mass spectrometry in the detection and analysis of primer-extended PCR products. The protocol involves PCR amplification of DNA using SNP specific primers, followed by a base extension reaction using the iPLEX chemistry (Sequenom). All SNP specific and mass extend oligonucleotides were designed using RealSNP and MassARRAY Assay Designer (Sequenom).

KEY RESEARCH ACCOMPLISHMENTS

Progress was made during the second year of this grant, although some of the work was delayed due to not being able to use the grant money until January 2008.

The preliminary results from the cytokine level analyses suggest that there is no significant association between IL-6 and TNF- α serum levels and breast density variables in postmenopausal women.

REPORTABLE OUTCOMES

Nothing yet. We recently started working on a manuscript on cytokine serum levels and breast density. As noted in the approved Statement of Work, dissemination of results is planned for the third year of the grant when most results will be available.

CONCLUSION

As noted above, our preliminary results suggest that there is no significant association between IL-6 and TNF-α serum levels and breast density. Based on published data showing that IL-6 and TNF-α regulate estrogen synthesis in the breast (5) and data showing that estrogens influence breast density (3, 4) we had originally hypothesized that among healthy women serum levels of IL-6 and TNF-α would be positively associated with percent and absolute breast density.

REFERENCES

1. McCormack VA, dos Santos Silva I. Breast density and parenchymal patterns as markers of breast cancer risk: a meta-analysis. Cancer Epidemiol Biomarkers Prev 2006;15:1159-1169.
2. Vachon CM, Brandt KR, Ghosh K, et al. Mammographic breast density as general marker of breast cancer risk. Cancer Epidemiol Biomarkers Prev 2007;16:43-49.

3. Rutter CM, Madelson MT, Laya MB, Segers DJ, Taplin S. Changes in breast density associated with initiation, discontinuation, and continuing use of hormone replacement therapy. JAMA 2001;285:171-176.
4. Cuzick J, Warwick J, Pinney E, Warren RM, Duffy SW. Tamoxifen and breast density in women at increased risk of breast cancer. J Natl Cancer Inst 2004;96:621-6218.
5. Purohit A, Newman SP, Reed MJ. The role of cytokines in regulating estrogen synthesis: implications for the etiology of breast cancer. Breast Cancer Res 2002;4:65-69.

APPENDICES

None